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Redescription of *Besnoitia tarandi* (Protozoa: Apicomplexa) from the reindeer (*Rangifer tarandus*)☆

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Abstract

Besnoitia tarandi tissue cysts were found in naturally-infected reindeer (*Rangifer tarandus*) from Finland. Infectivity of its tissue cysts, bradyzoites, and tachyzoites to animals and cell culture was studied. The bradyzoites and tissue cysts were not infectious to out-bred mice, rabbits or gerbils. When fed tissue cysts, neither cats nor dogs excreted oocysts. However, the parasite was lethal to interferon-gamma gene knock out mice irrespective of the route of inoculation. The parasite was grown successfully in African Green Monkey cells from tissues of two reindeer for the first time. Non-dividing, uninucleate tachyzoites from smears from cell cultures were 5.6×1.4 μm (4.5–7.4×1.0–1.9, $n=50$) in size. Longitudinally-cut bradyzoites in tissue sections measured 7.4×1.3 μm (6.5–7.8×1.0–1.6, $n=30$). Ultrastructurally, tachyzoites and bradyzoites were similar to those in other *Besnoitia* species, and in particular to parasites described from cattle (*Besnoitia besnoiti*) and equids (*Besnoitia bennetti*) in that their bradyzoites lacked enigmatic bodies. Based on comparative analysis of three portions of nuclear ribosomal DNA (the small and large subunits and the first internal transcribed spacer) *B. tarandi* was found to be more closely related to the other congeners described from ungulates. The parasite was formally redescribed and specimens deposited in the US National Parasite Collection.
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Keywords: *Besnoitia tarandi*; Finland; Mice; Gerbils; Cell culture; Ultrastructure; Sequence analysis

1. Introduction

Species of the coccidian genus *Besnoitia* parasitise cattle, goats, equids, reindeer, caribou, opossums, rabbits, rodents, and lizards (Leighton and Gajadhar, 2001; Dubey et al., 2003a). To date, nine species in the genus have been named: *Besnoitia bennetti, Besnoitia jellisoni*, *Besnoitia wallacei*, *Besnoitia tarandi*, *Besnoitia darlingi*, *Besnoitia caprae*, *Besnoitia besnoiti* (type species), *Besnoitia akadoni* and *Besnoitia oryctofelisi* (Dubey et al., 2003a, 2003c). However, considerable uncertainty exists regarding the identity of some of these species because the life cycles of only three (*B. darlingi*, *B. wallacei*, and *B. oryctofelisi*) of these species are known, and morphological differences among the remaining species are poorly defined (Dubey et al., 2003a). Although parasites isolated from distinct intermediate host species have traditionally been presumed to represent distinct parasite taxa, it will be difficult to test their actual host specificity until natural definitive hosts are identified.

Besnoitia tarandi was first reported from reindeer (*Rangifer tarandus tarandus*) and caribou (*Rangifer tarandus caribou*) from Alaska, USA by Hadwen (1922), who named it as *Fibrocystis tarandi* n. sp. Levine (1961) transferred it to the genus *Besnoitia*. Subsequently, infections

☆ Nucleotide sequence data reported in this paper are available in the Genbank, EMBL and DDBJ databases under the accession numbers: AY616163, AY616164, AY665400.

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in reindeer, caribou, mule deer, roe deer, and musk ox were attributed to this parasite (Nikolaevskii, 1961; Nordkvist, 1966; Choquette et al., 1967; Skjenneberg and Slagsvold, 1968; Wobeser, 1976; Rehbinder et al., 1981; Lewis, 1989, 1992; Glover et al., 1990; Hilali et al., 1990; Ayroud et al., 1995; Luco et al., 2000; Leighton and Gajadhar, 2001).

The life cycle of *B. tarandi* is not yet completely known. Only the tissue cyst and bradyzoite stages are known, and their structures are not fully described. Thus, little basis exists to evaluate whether the same etiological agent establishes infection, and induces disease, in all these hosts. Here, we report the successful isolation of *B. tarandi* in culture and in immunodeficient mice, describe the structure of its bradyzoites and tachyzoites, and define portions of its ribosomal DNA to aid its diagnosis and comparison to related coccidia. Specimens are also deposited in museum collections to aid future comparison.

2. Materials and methods

2.1. Naturally-infected reindeer

Grossly visible *Besnoitia* sp. tissue cysts were observed in 19 of the 735 reindeer examined at necropsy at the National Veterinary and Food Research Institute, Regional Unit, Oulu, Finland (EELA), between January 1999 and February 2004 (Table 1). Approximately 2 kg of infected muscles and periosteum from reindeer no. 16 and an entire hind leg from animal no. 18 (Table 1) were shipped from Finland to the USDA laboratory in Beltsville, MD for further study. The carcasses were received at Beltsville in 7 (reindeer no. 16) or 13 (reindeer no. 18) days after the killing of the reindeers.

2.2. Infection of laboratory animals

Laboratory-reared dogs, gerbils, rabbit, mice, and cats were inoculated with *B. tarandi*. Animal experiments were performed according to Animal Care Protocols of the US Department of Agriculture.

The dogs were obtained from Covance Research Products Inc., Denver, CO, USA. The cats were from a parasite-free colony (Dubey, 1995). Muscles and periosteum heavily infected with tissue cysts from reindeer no. 16 were fed to two dogs and two cats. The dogs consumed approximately 1 kg of infected tissues and the cats consumed at least 50 g of infected material in addition to tissue cysts that were separated from the periosteum. About 200 g of infected tissues from reindeer no. 18 were fed to two additional cats. Faeces of cats and dogs were examined for coccidian oocysts by sugar flotation daily for 30 days thereafter (Dubey, 1995).

The five to eight week old female gerbils (*Meriones ungulatus*) were obtained from Charles River Laboratory, Stoneridge, NY, USA. The 20–25 g female Swiss Webster (SW) out-bred mice were obtained from Taconic Farms, Germantown, NY, USA. The five to 10 week old female interferon-gamma gene knockout (KO) mice were obtained from Jackson Laboratories; these knockout mice are highly susceptible to intracellular parasites because they lack the capacity to produce the cytokine interferon-gamma, necessary for intracellular immunity (Dubey and Lindsay, 1998).

Muscle and periosteum, containing visible tissue cysts from reindeer no. 16 were homogenised in phosphate buffer saline (PBS) by mortar and pestle. Rodents were inoculated with either the undigested homogenate or with homogenate incubated with 0.5% trypsin for 30 min at 37 °C, washed and resuspended in antibiotics (1,000 units of penicillin and 100 μg of streptomycin). Details of rodents used to study infectivity of this *Besnoitia* are given in Table 2. In an attempt to clone the parasite, a single tissue cyst was separated from the host tissues on a glass slide and incubated with acidic-pepsin (Dubey, 1998) for 10 min to soften the outer tissue cyst wall. The bradyzoites were released by mechanical disruption of the tissue cyst, and inoculated s.c. in to three KO mice (Table 2). Parasites recovered from the liver of one KO mouse that died subsequently, were seeded to culture (see Section 2.3). From reindeer no. 18, pieces of connective tissue containing numerous tissue cysts were washed in antibiotic solution, and homogenised. The released bradyzoites were filtered through a 5-μm filter, and inoculated on to cell cultures and in mice and gerbils (Table 2).

An eight week-old New Zealand White rabbit (no. 1) was inoculated s.c. and intramuscularly with 2×10^6 cell culture-derived *B. tarandi* tachyzoites, and 56 days later with another dose of 1×10^6 tachyzoites. The rabbit was bled and euthanised 100 days after the initial inoculation.

2.3. In vitro cultivation

Bradyzoites released from tissue cysts from both reindeers were cultured in vitro. An aliquot of the trypsin treated muscle homogenate from reindeer no. 16 (see earlier) was seeded onto culture flasks containing monolayers of African Green Monkey (CV-1) cells, and M 617 cells (Dubey et al., 2002). To study conversion of bradyzoites to tachyzoites, bradyzoites released from tissue cysts from reindeer no. 18 were seeded on to CV-1 cells grown on coverslips. Coverslips were removed at 1, 4 h or at each 24-h interval, fixed in Bouin's, stained with Giemsa, and examined microscopically. Organisms were measured using the motorised system microscope (Olympus Optical Co., Tokyo, Japan) with a Olympus FV camera and Olympus Microsuite™ B3SV software. Cultures of CV-1 were also infected with a clone obtained from the liver of a KO mouse infected with a single tissue cyst. Serial 10-fold dilutions of tachyzoites of this clone were injected into KO mice to study the infectivity. Cloned tachyzoites were also used to infect SW mice (Table 2).

Table 1
Reindeer with *Besnoitia tarandi* necropsied at FELA, Finland

Animal No.	Month/ Year	Age	Submitted tissues	Symptoms/cause of death	Tissues with *B. tarandi* tissue cysts
1	03/1999	3 years[a]	Skin		Subcutaneous tissue
2	10/1999	5 months[b]	Skinned carcass without head	Weakness/glossitis and pleuropneumonia (*Fusobacterium necrophorum*)	Muscle fascia
3	11/1999	Adult[b]	Whole reindeer	Killed because of weakness/peritonitis and granulomatous lesions in various organs caused by parasites (*Setaria tundra*)	Subcutaneous tissue and muscle fascia
4	12/1999	1.5 years[c]	Whole reindeer	Wet belly, weight loss/fibrinous pericarditis, erosive stomatitis, pharyngitis and abomasitis	Subcutaneous tissue, sclera, conjunctiva, muscle fascia, respiratory mucosa, peritoneum, lymph node
5	01/2000	7 years[b]	Whole reindeer	Killed because of weakness/ulcerative stomatitis, septicaemia	Subcutaneous tissue
6	01/2000	Adult[b]	Whole reindeer	Central nervous symptoms/periodontitis, myositis, myelitis (*Fusobacterium necrophorum*)	Subcutaneous tissue, muscle fascia
7	03/2000	2 years[b]	Whole reindeer	Weight loss/ulcerative stomatitis and rumenitis, peritonitis and septicaemia	Subcutaneous tissue
8	10/2000	5 months[c]	Formalin fixed tissue (muscle, muscle fascia, skin, lymph node, lung)		Muscle fascia, muscle, skin, lymph node, lung
9	11/2000	5 months[c]	Skin, head, metacarpus	Emaciation, skin disease	Subcutaneous tissue, skin, periosteum
10	11/2000	Adult[b]	Head	Opacity in left eye/persistent pupillary membrane	Subcutaneous tissue, sclera, conjunctiva, periosteum
11	01/2001	Adult[b]	Whole reindeer	Killed because of weakness/foot rot, generalised besnoitiosis	Subcutaneous tissue, muscle fascia, periosteum, mucous membrane of mouth, pharynx and trachea, muscle, heart, lung, kidney, lymph node
12	03/2001	10 months[b]	Whole reindeer	Wet belly/emaciation	Subcutaneous tissue
13	09/2001	4 months[c]	Whole reindeer	Killed because of lameness and skin disease/generalised besnoitiosis	Subcutaneous tissue, skin, mucous membrane of mouth and respiratory track, sclera, conjunctiva, peritoneum, brain
14	12/2001	6 months[c]	Spleen, metacarpus, thigh muscle	Sample from meat inspection/granulomatous lesions in muscle caused by parasites (*Onchocerca* sp.)	Muscle fascia
15	10/2003	5 months	Pieces of forelimbs	Sample from meat inspection	Muscle fascia
16	11/2003	6 months[a]	Formalin fixed subcutaneous tissue	Sample from meat inspection	Subcutaneous tissue
17	11/2003	Adult[b]	Skinned forelimbs	Sample from meat inspection	Muscle fascia
18	01/2004	1.5 years[b]	Whole reindeer	Killed because of lameness/emaciation, generalised besnoitiosis	Subcutaneous tissue, skin, mucous membranes of mouth, pharynx and trachea, sclera, conjunctiva, nasal turbinate, periosteum
19	01/2004	5.5 years[c]	Whole reindeer	Weakness/emaciation, abscessed pharyngitis and septicaemia caused by bacteria (*Fusobacterium necrophorum, Arcanobacterium pyogenes*)	Subcutaneous tissue

[a] Sex not known.
[b] Female.
[c] Male.

2.4. Necropsy and histopathological examination

Samples of brain, lung, heart, tongue, liver, kidney, intestine, mesenteric lymph node, urinary bladder, and limb muscle of dogs, gerbils, rabbit, mice, and cats were fixed in 10% neutral buffered formalin. Paraffin-embedded sections were cut at 5 μm, and examined after staining with H and E.

2.5. Transmission electron microscopy

For TEM, tissues were fixed in Karnovsky fixative, glutaraldehyde or in 10% buffered neutral formalin. They were subsequently post-fixed in osmium and processed for TEM. For the study of tachyzoites, we used CV-1 cells infected in vitro and the livers of KO mice 10 and

Table 2
Infectivity of *Besnoitia tarandi* to mice and gerbils

Source of inoculum	Animal species	No.	Inoculum	Route	Outcome[a]	*B. tarandi* in tissues[b]
Reindeer No. 16	KO mice	15	Digested tissues[c]	s.c.	D 9–21	Yes
		5	Undigested tissues[d]	s.c.	D 9–13	Yes
		2	Undigested	oral	D 14	Yes
		23	Tachyzoites[e] $1–10^7$	s.c.	D 7–16	Yes
	SW mice	2	Tissue cysts	s.c.	S 49	No
		5	Tachyzoites[e] 10^6	s.c.	S 34 or 45	No
	Gerbils	3	Digested tissues	s.c.	S 35 or 59	No
		2	Tachyzoites[e]	s.c.	S 60	No
		2	Tachyzoites[e]	i.p.	S 40	No
Reindeer No. 18	KO mice	5	Bradyzoites	s.c.	D 10–14	Yes
		7	Bradyzoites	oral	D 11–14	Yes
		2	Tachyzoites[e]	s.c.	D 10	Yes
	Gerbils	3	Bradyzoites	s.c.	S 37	No

[a] D=died or killed when ill. S=survived not ill.
[b] By direct examination and by immunohistochemistry.
[c] Homogenised tissues digested in trypsin; No. of organisms in inoculum unknown.
[d] Homogenised tissues not digested; No. of organisms in inoculum unknown.
[e] From cell culture.

11 days p.i. The ultrastructure of three formalin-fixed tissue cysts from reindeer nos. 13 and 16 was also studied.

2.6. Immunohistochemical staining

For immunohistochemical staining, two types of antibodies were used at 1: 10,000 serum dilution. The polyclonal anti-*B. tarandi* antibody was from rabbit no. 1 (see Section 2.2). The bradyzoite-specific rabbit antibody (BAG-1, also called BAG-5) directed against a heat-shock protein from *Toxoplasma gondii* was supplied by McAllister et al. (1996). Staining was performed as described previously (Dubey and Sreekumar, 2003).

2.7. Molecular systematics

DNA from *B. tarandi* bradyzoites and cloned tachyzoites was extracted by digestion, ethanol precipitation, and purification using Qiagen DNAeasy columns according to the manufacturer's protocol. Aliquots, along with extraction negatives, were subsequently used as templates in polymerase chain reaction (PCR) assays to characterise portions of the small and large subunits of nuclear ribosomal DNA, as well as the first internal transcribed spacer 1 (ITS-1). These products were directly sequenced on an ABI 3100 fluorescent sequencer using BigDye 3.1 terminator chemistries. Sequence contigs were assembled and edited using ContigExpress (VectorNTI, Informax Inc.) and subsequently aligned with other available homologues using ClustalW. The phylogenetic position of *B. tarandi* was subsequently estimated from Kimura 2-parameter distances using 500 bootstrap replications under the minimum evolution objective criterion as implemented by MEGA v. 2.1 (Kumar et al., 2001).

3. Results

3.1. Structure of tissue cysts and bradyzoites

Tissue cysts were located in the skin, subcutaneous tissue, conjunctiva, sclera, periosteum of long bones and the skull, muscle fascia, and occasionally in soft tissues (Table 1, Fig. 1). They were white to glistening white and embedded in host tissue. Tissue cyst walls were up to 50 μm thick (Fig. 2). The thickness of the tissue cyst wall varied. Under the light microscope three layers were recognised. The outer layer (layer I) consisted of connective tissue up to 30 μm thick; with H and E it stained darker than the middle layer. The middle layer (layer II) was 5–20 μm thick depending on the shrinkage during fixation; it had pseudopod-like extensions (Fig. 2D) and enclosed host cell elements including host cell nuclei. The innermost layer (layer III) was less than 1 μm thick and enclosed bradyzoites. The bradyzoites measured 6.5×1.3 μm (5.0–7.7×0.9–1.6, $n=50$) in smears. Longitudinally cut bradyzoites in 1-μm Toluidine blue sections measured 7.4×1.3 μm (6.5–7.8×1.0–1.6, $n=30$). Tissue cysts were not septate. Occasionally, two tissue cysts were enclosed in the same host cell (Fig. 1E, F), giving the false impression of septa.

The three layers seen under light microscopy were also recognised by TEM (Fig. 3A). The outer layer consisted of connective tissue. The middle layer contained host cell nuclei and an accumulation of endoplasmic reticula; filamentous extensions were seen that projected towards the outer most layer (Fig. 3A). The innermost layer consisted of a thin parasitophorous vacuolar membrane, which was lined by an amorphous granular layer, which varied in thickness. Numerous bradyzoites were enclosed in the granular layer. Bradyzoites varied in size and shape (Figs. 2–4), some were plump whereas others were

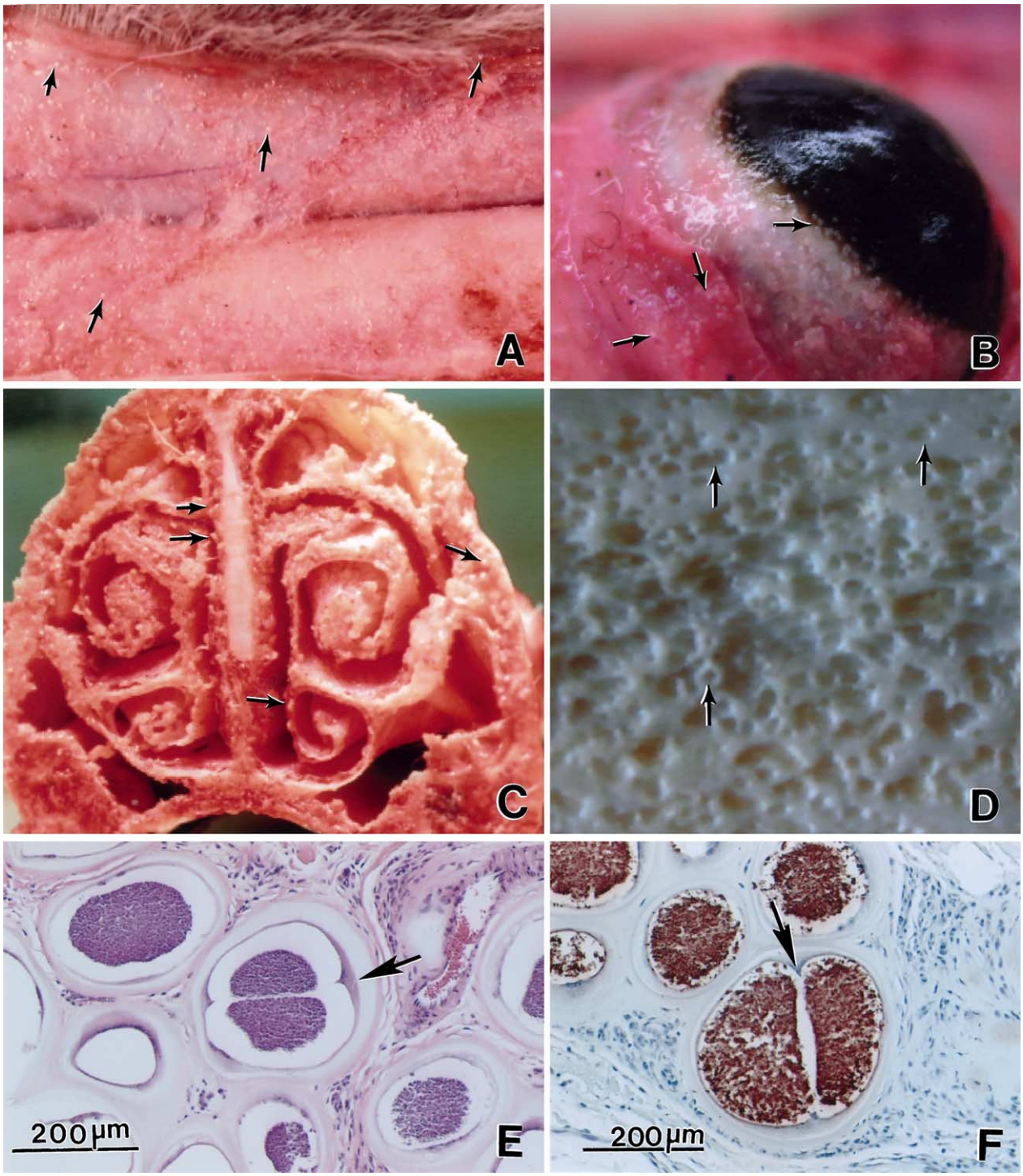

Fig. 1. *Besnoitia tarandi* tissue cysts (arrows) in naturally-infected reindeer. (A–D) Gross appearance of tissue cysts (A) in periosteum of tibia and fibula (B) cornea and sclera of eye, (C) turbinates, and (D) impressions of tissue cysts on bone, unstained. (E and F) Sections of tissue cysts in muscle and fasciae. (E) Haematoxylin-eosin. (F) Immunostaining (brown colour) with *B. tarandi* antibodies. Note two tissue cysts (arrows) enclosed in one host cell.

slender (Fig. 3C). Ten longitudinally cut bradyzoites were 4.5–7.0×1.13–1.53 µm in size on TEM sections (Fig. 4). A few organisms were seen dividing into two zoites by endodyogeny (Fig. 4E). The bradyzoite pellicle was lined by an electron-dense material at the conoidal end (Fig. 4). Bradyzoites contained a conoid, micronemes, rhoptries, a nucleus, amylopectin, a mitochondrion, and dense granules. Micronemes were few (not more than 50 per section) and were mostly located at the conoidal end, but some extended to the posterior (non-conoidal) end. They were up to 210 nm long and approximately 35 nm wide. The rhoptries extended up to the posterior end (Fig. 4).

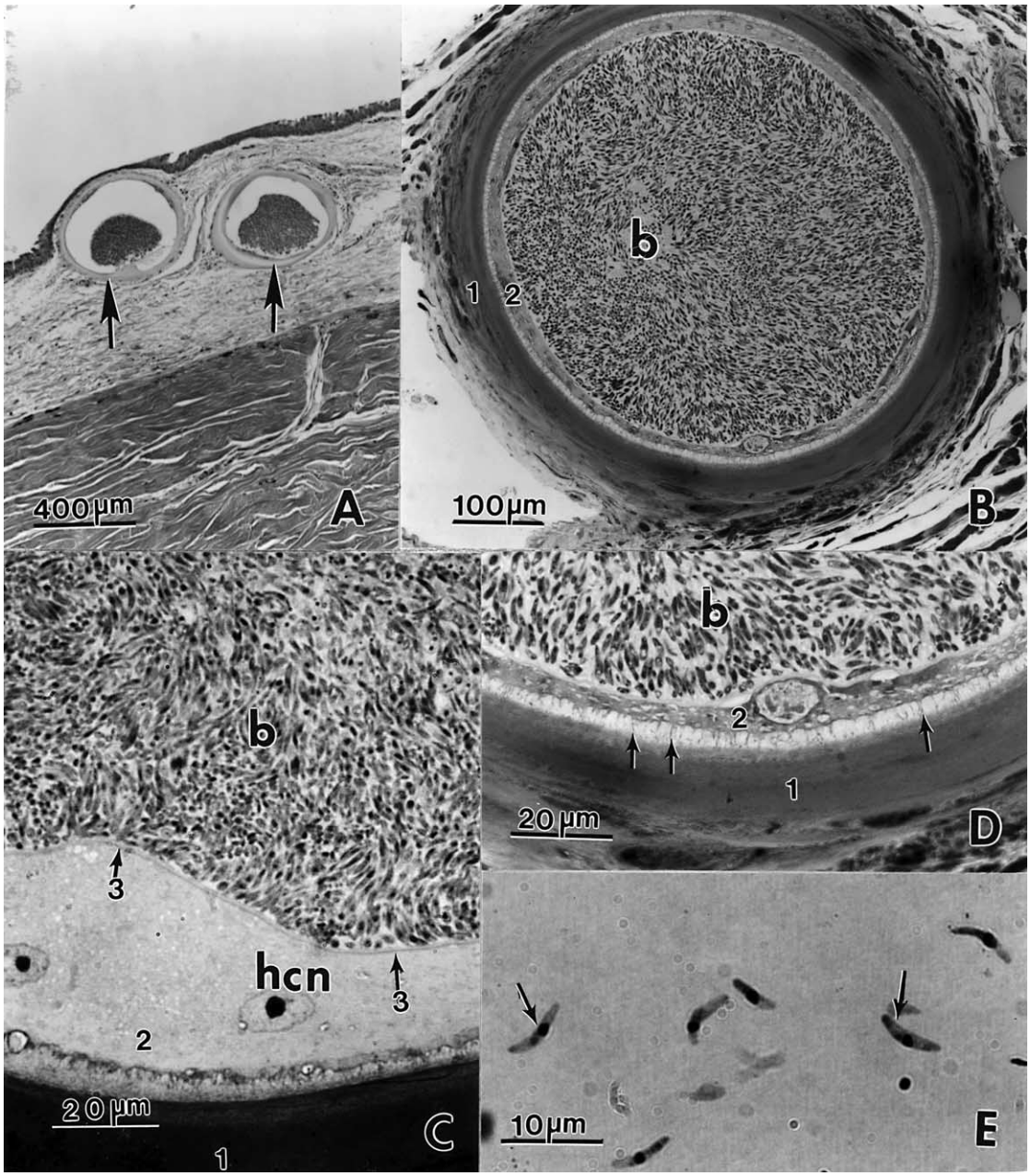

Fig. 2. Tissue cysts and bradyzoites of *Besnoitia tarandi*. (A) Two tissue cysts (arrows) in section of conjunctiva. Haematoxylin-eosin. (B–D) Cross section of tissue cysts in fascia. Toluidine blue. There are three layers, outer (1) thick layer, a middle layer (2) and a third (3) thin layer that is not always visible at low magnification. Numerous bradyzoites (b) are enclosed in layer 3. Note host cell nuclei (hcn) enclosed in layer 2, which has pseudopod-like projections (arrows in D). (E) Bradyzoites (arrows) released from a tissue cyst in a smear stained with Giemsa.

The contents of rhoptries were electron-dense; in some sections, the interior of the rhoptries had shrunk, giving it the appearance of enigmatic bodies (Fig. 4). The bulbous ends of rhoptries were approximately 35 nm in diameter. Occasionally, the bulbous end was turned towards the conoidal end of the bradyzoite (Fig. 4B). A maximum of five rhoptries were seen in a given section. The position of the nucleus in bradyzoites was central to terminal (Fig. 4). Enigmatic bodies were not found in any of the bradyzoites examined from three tissue cysts (Figs. 3 and 4).

3.2. Isolation of B. tarandi in cell culture

Plaques were recognised in CV-1 monolayers six days after inoculation with trypsinised reindeer tissues. Tachyzoites were observed in cells adjacent to the plaques. Tachyzoites from this culture were preserved in liquid nitrogen 17 days after this culture had been originally seeded. Tachyzoites were also grown in CV-1 cells inoculated with tissues of a KO mouse that had died of besnoitiosis 10 days after inoculation with reindeer tissues.

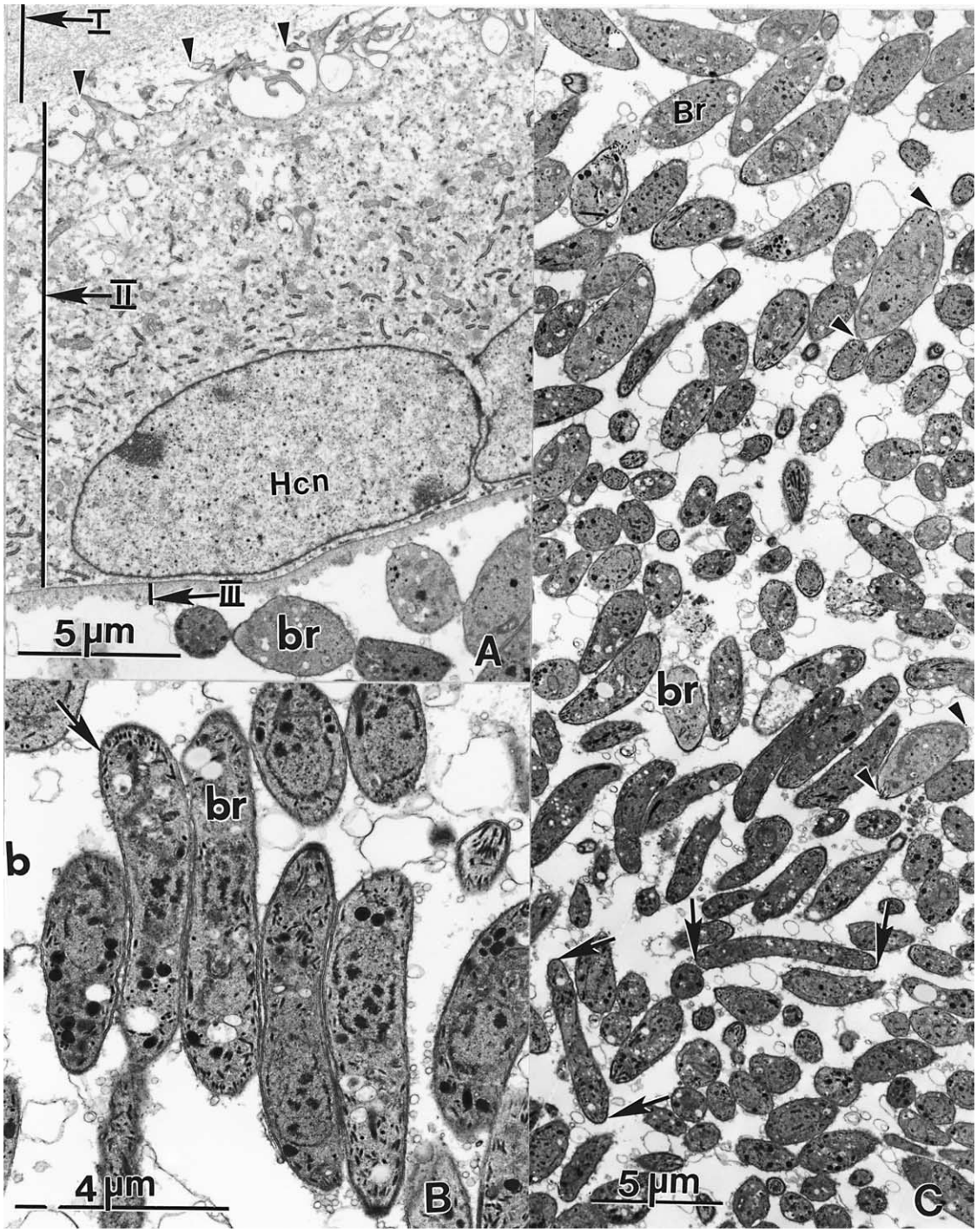

Fig. 3. TEM of tissue cysts of *Besnoitia tarandi*. (A) Note 3 tissue cyst wall layers (I, II, III), host cell nuclei (Hcn) in layer II, which shows pseudopod-like projections (arrowheads), and bradyzoites (br) below layer III. (B, C) Note different shapes and sizes of bradyzoites, slender (arrows) and plump (arrowheads).

In coverslip cultures infected with bradyzoites and stained sequentially, the first intracellular forms were seen in 4 h. After 20 h, many intracellular forms were noticed and some were enlarged, with large vesicular nuclei, indicating initiation of endodyogeny. By 48 h, most of the infected cells had inclusions containing four, while some had rosettes of eight tachyzoites. (Fig. 5A, B). In the coverslip removed after 72 h, most of the infected cells were packed with more than 32 parasites. Rupture of infected cells and release of tachyzoites into the extracellular fluid was seen by 96 h. By day 8 p.i., more than 50% of the monolayer was destroyed. Parasites grew for several generations and divided by simultaneous endodyogeny, sometimes forming a residual body at the center of rosettes (Fig. 5C, D). Extracellular forms were more lunate than the intracellular forms (Fig. 5D, E). The size of tachyzoites

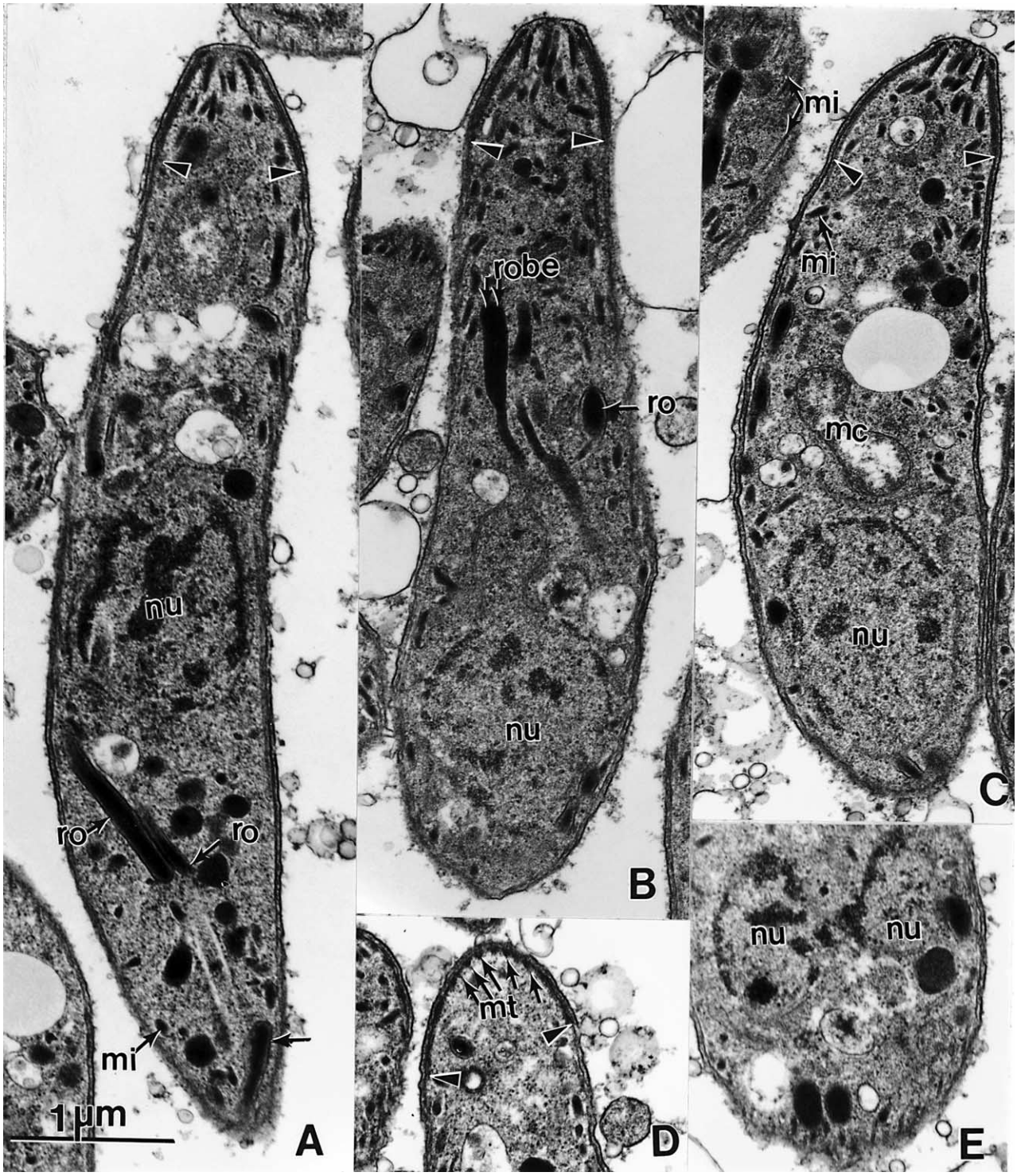

Fig. 4. Longitudinal sections of three bradyzoites with different shapes and sizes (A–C) and (D) conoidal, and (E) non-conoidal ends of two other bradyzoites. Note (A, B) elongated rhoptries with bulbous end of one rhoptry (robe) pointed towards the conoidal end (B). Some of the rhoptries (ro) appear to have a shrunken core. Also note the (A) central and (B, C) terminal position of the nuclei, thickening of the pellicle at the conoidal end (arrowheads), micronemes (mi), mitochondrion (mc), microtubules (mt) and (E) two nuclei (nu). Bar in (A) applies to all parts.

varied, depending at the stage of division. Dividing organisms were 6.1×2.8 µm (4.0–8.3×1.7–3.7, $n=30$). Extracellular tachyzoites were 5.6×1.4 µm (4.5–7.4×1.0–1.9, $n=50$). The nucleus in tachyzoites was located centrally and was often vesicular.

3.3. Ultrastructure of tachyzoites

Tachyzoites were located in a parasitophorous vacuole containing tubular network, in the host cell cytoplasm (Fig. 6). A few tachyzoites were located in the endothelium

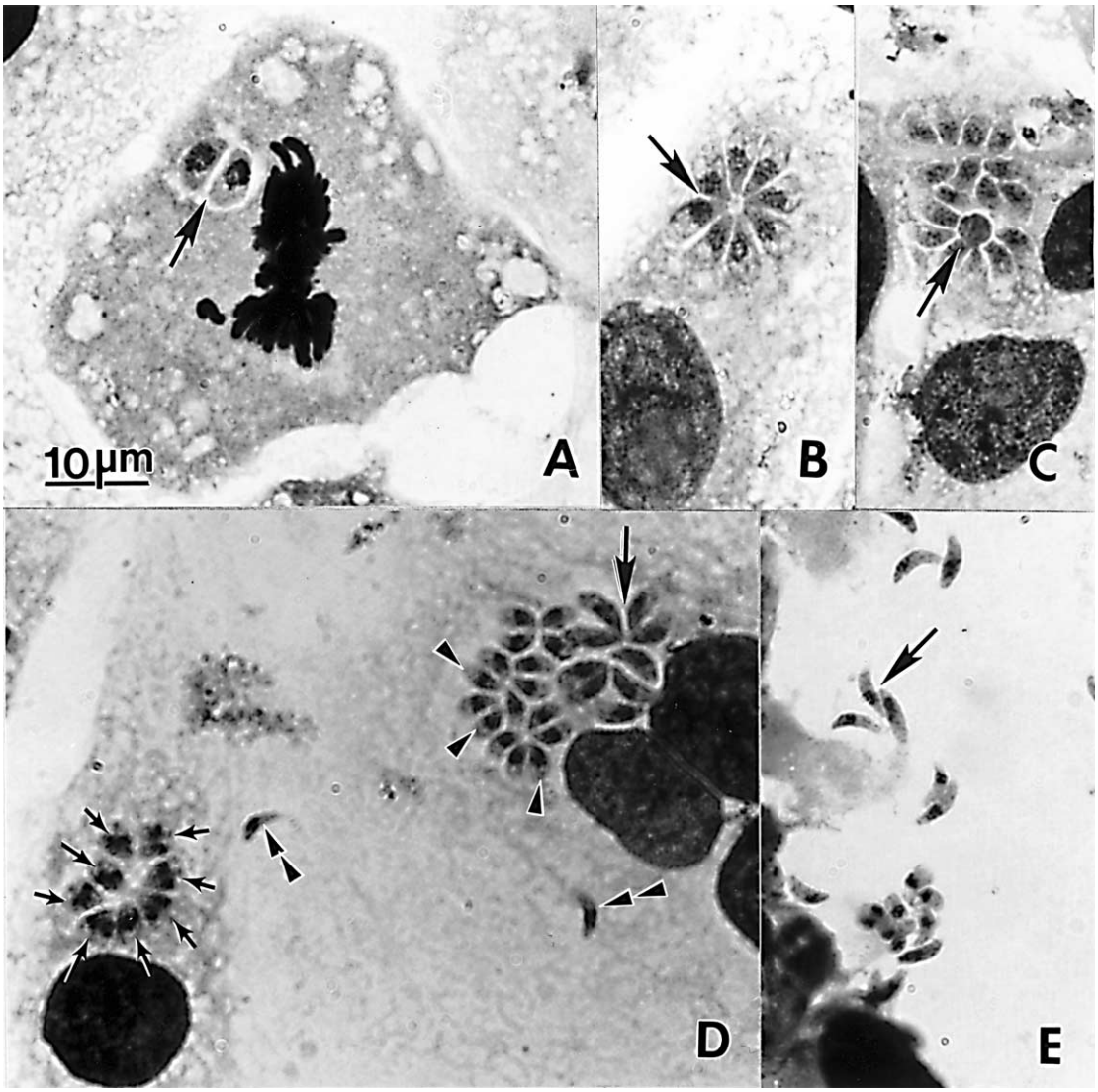

Fig. 5. Tachyzoites of *Besnoitia tarandi* in CV-1 cells. Giemsa. Bar applies to all parts. (A) Two tachyzoites (arrow) in a vacuole, 20 h p.i. (B) A rosette of eight tachyzoites (arrow), 48 h p.i. (C) A group of tachyzoites with a residual body (arrow). (D) Tachyzoites with varying shapes and sizes. Note eight tachyzoites (small arrows) in simultaneous division, two groups (large arrow, arrowheads) of intracellular tachyzoites of different sizes, and two extracellular lunate tachyzoites (double arrowheads). (E) Extracellular lunate tachyzoites (arrow).

and intravascular leucocytes (Fig. 6A). Tachyzoites divided by endodyogeny (Fig. 6B). They contained a conoid, a micropore, several micronemes, rhoptries, mitochondrium, and a centrally located nucleus, but no amylopectin granules (Fig. 6). The micronemes were located anterior to the nucleus and often arranged in rows (Fig. 6D). The rhoptries were few in number and their contents were electron-dense. Rhoptries extended posterior to the nucleus (Fig. 6D). An unidentified membraned body was present at the conoidal end (Fig. 6D).

3.4. Immunohistochemistry

The innermost layer (third layer) and the enclosed bradyzoites in tissue cysts stained positively with anti-*B. tarandi* antibodies (Fig. 1F). Bradyzoites, but not the tachyzoites, were stained with BAG-1-antibodies. Tachyzoites reacted positively with anti-*B. tarandi* antibodies, but not with the BAG-1 antibodies.

3.5. Infection of laboratory animals

No oocysts were seen in faeces of either the dogs or cats nor were any protozoa identified in the histological sections of these animals.

All gerbils and out-bred mice remained asymptomatic during the period of observation (35–60 days p.i), whether inoculated with reindeer tissues or with tachyzoites derived from cell culture or from mice. Protozoa was not identified in the tissues of these animals using immunohistochemical staining with *B. tarandi* antibodies (Table 2).

All KO mice inoculated s.c. with *Besnoitia* parasites from reindeers died of acute besnoitiosis between 9 and 26 days

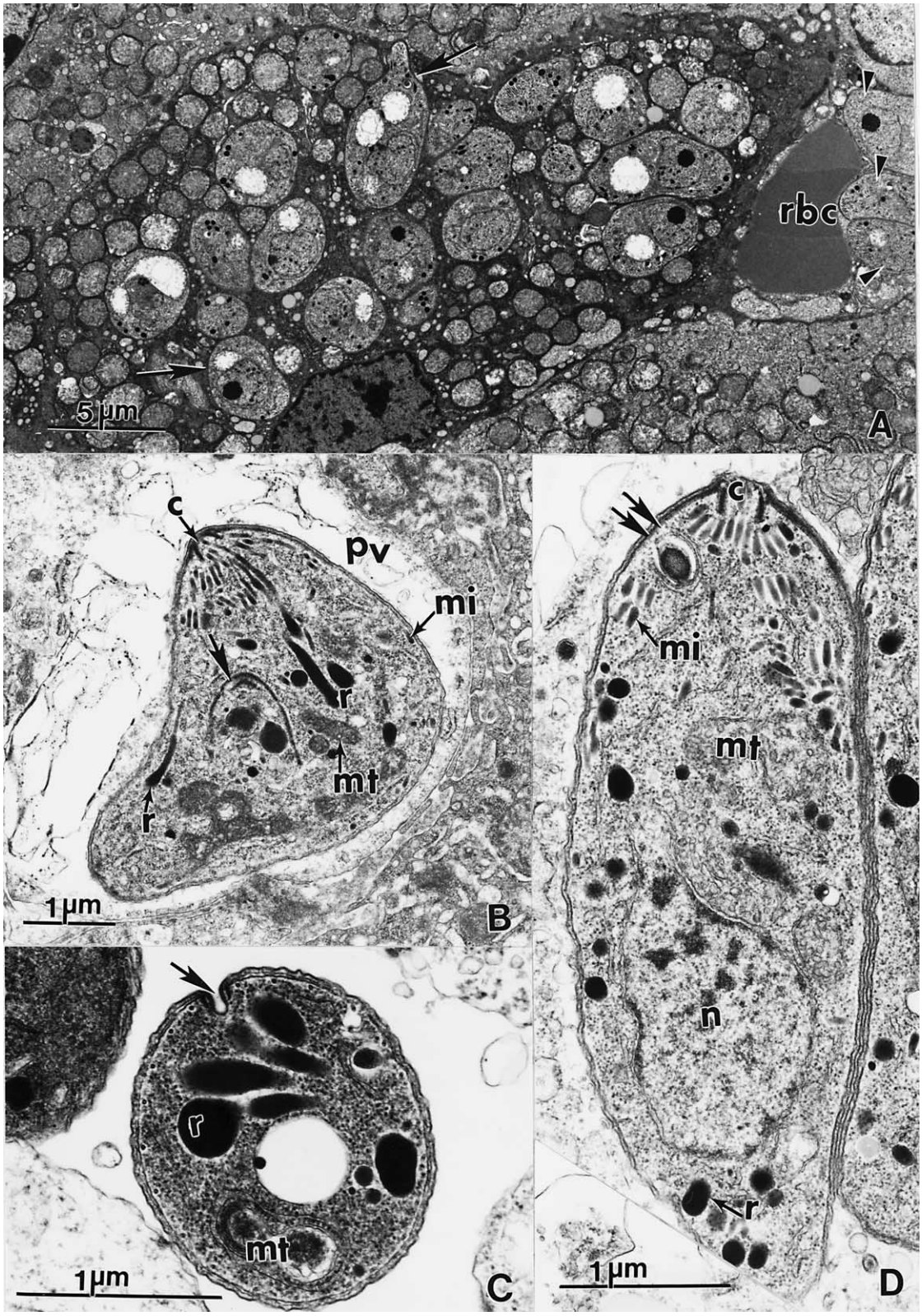

Fig. 6. TEM of *Besnoitia tarandi* tachyzoites (A, B) from liver of a KO mouse 11 days p.i., and (C, D) from cell culture. (A) Numerous tachyzoites (arrows) apparently free in the cytoplasm of a hepatocyte. Note three tachyzoites (arrowheads) in the lumen of a capillary adjacent to a red blood cell (rbc). (B) A dividing tachyzoite located in a parasitophorous vacuole (pv) in the cytoplasm of a hepatocyte. Note conoid (c), micronemes (mi), rhoptries (r) and mitochondrium (mt) of the mother cell and anlagen (arrow) of a daughter zoite. (C) Cross section of a tachyzoite showing a micropore (arrow), rhoptries (r), and mitochondrium (mt). (D) Longitudinal section of a tachyzoite showing conoid (c), micronemes (mi), rhoptries (r), elongated mitochondrium (mt), nucleus (n), and an unidentified membraned structure (double arrows) at the conoidal end.

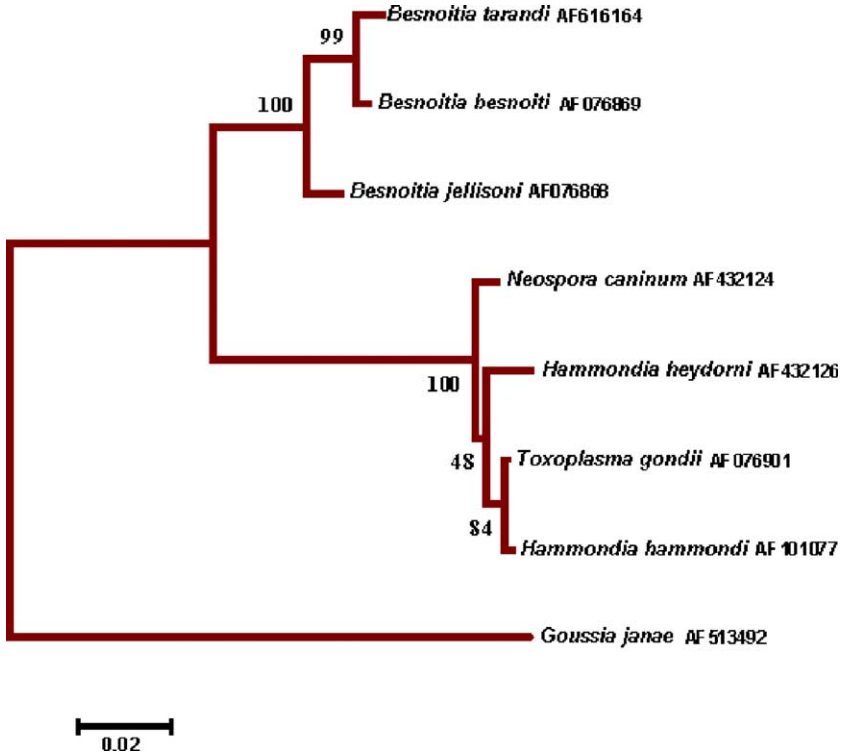

Fig. 7. Consensus of 500 bootstrap replicate trees reconstructed under the minimum evolution criterion based on Kimura 2-parameter distances, using an alignment composed of 516 bp of the 28S rDNA.

p.i. and tachyzoites were found in their tissues. Tachyzoites were more abundant in the liver, lungs, kidneys, adrenals and spleen and were rare in the brain. They were located in many cell types, including blood vessels.

The KO mice inoculated orally with free bradyzoites or tissue cysts died of acute besnoitiosis 8–12 days p.i. Tachyzoites were seen in many organs, but were most abundant in the liver, lungs and spleen.

One of the three KO mice inoculated with bradyzoites freed from a single tissue cyst died of besnoitiosis on day 21 p.i., and numerous tachyzoites were found in its peritoneal exudate. The KO mice inoculated with a 10-fold serial dilution (two mice per dilution) of tachyzoites estimated to contain fewer than 10 organisms died of besnoitiosis with demonstrable tachyzoites; organisms derived from this mouse were considered to be clonal because they were from a single tissue cyst and had been further end titrated.

Neither protozoa nor lesions were found in the rabbit inoculated with tachyzoites.

3.6. Molecular systematics

The sequences of small (GenBank AY616163) and large (GenBank AY616164) subunits of the nuclear ribosomal DNA of *B. tarandi* were compared with those of other *Besnoitia* spp., as well as other apicomplexans. Both the large and small subunit rDNA trees provided strong support that *Besnoitia* spp. comprise a monophyletic group that is most closely related to a clade including parasites assigned to the genera *Toxoplasma*, *Neospora*, and *Hammondia* (Figs. 7 and 8).The comparatively greater variability in the ITS-1 portion of rDNA (AY665400) provided a more informative basis to explore diversity within the genus, providing strong evidence that a particularly close relationship exists among the *B. tarandi* and the causative agents of bovine, caprine, and equine besnoitiosis (Fig. 9).

3.7. Taxonomic summary

Intermediate type host: Reindeer (*Rangifer tarandus tarandus*).

Other unconfirmed intermediate hosts: Caribou (*Rangifer tarandus caribou*), mule deer (*Odocoileus hemionus hemionus*), musk ox (*Ovibos moschatus*) and roedeer (*Capereolus capereolus*).

Definitive host: Unknown.

Locality: North America, Fennoscandia, former USSR.

Specimens deposited: Hapantotypes were deposited in the United States National Parasite Collection (USNPC), United States Department of Agriculture, Beltsville, MD, USA: (a) histologic sections of tissue cysts from subcutaneous tissue of naturally-infected reindeer no. 18, stained with H and E (USNPC No. 94803), stained with anti-*B. tarandi* antibodies (USNPC No.94804), 1-um Toluidine blue (USNPC No. 94805) and unstained tissue in 10% buffered neutral formalin (USNPC No. 94806) (b) histologic sections of tissues of KO mice experimentally-infected with tachyzoites, 11 days p.i. stained with H and E (USNPC No. 94807) and unstained

Fig. 8. Consensus of 500 bootstrap replicate trees reconstructed under the minimum evolution criterion based on Kimura 2-parameter distances, using an alignment composed of 940 bp of the 18S rDNA.

tissues in formalin (USNPC No.94808) and (c) tachyzoites in cell culture preparations of CV-1 cells stained with Giemsa (USNPC No.94809).

Live cultures of tachyzoites of *B. tarandi* were deposited in the American Type Culture Collection (ATCC), Manassas, Virginia 20108, USA, ATCC Accession No. PRA-126.

4. Discussion

Until recently, little was known of the biology of *B. tarandi*. Glover et al. (1990) provided a detailed description of an outbreak of besnoitiosis in captive caribou, mule deer, and reindeer in Assinibione Park Zoo, Winnipeg, Manitoba, Canada. Infection appeared to have spread from caribou to mule deer and reindeer. In total 28 caribou, 10 mule deer, and three reindeer died or were euthanised. Lesions of besnoitiosis were similar in caribou and mule deer. Tissue cysts were seen in ulcerative nasal and pharyngeal epithelium, skin, ocular sclera, tendons, periosteum, and rarely on visceral serosa. Lesions in reindeer apparently were similar to those in caribou and mule deer but were not described. Tissue cysts were not infective to Chinese water deer (*Hydropotes inermis*), one mule deer (*O. hemionus hemionus*), one white tailed deer (*Odocoileus virginianus*), one Formosan sika deer (*Cervus nippon taiouanus*) or to laboratory mice. *Besnoitia*-like oocysts were not found in the faeces of two raccoons (*Procyon lotor*), one cat (*Felis domesticus*) or two foxes (*Vulpes vulpes*) fed *Besnoitia*-infected tissues.

Ayroud et al. (1995) conducted the first detailed study of tissue localisation of *Besnoitia* tissue cysts in five naturally-infected reindeer that had acquired infection at the same zoo as reported by Glover et al. (1990). The reindeers were 2, 3, 7, 8, and 9 years old. They were euthanised and virtually all of their tissues were studied histologically. *Besnoitia* sp. tissue cysts were found in skin, sclera, nasal turbinates, periosteum, tendons, tendon sheaths, corium of the hoof, testicular tunis and intima of the blood vessels. Tissue cysts were present in the blood vessels of the turbinates. No tissue cysts were found in other organs including liver, lungs, kidneys, brain, spleen, lymph nodes, and the gastrointestinal tract. Tissue cysts measured up to 400 μm in diameter. Ultrastructurally, bradyzoites were 8–10× 2–2.6 μm in size, contained two to four rhoptries, 30 or more micronemes, but they did not report the presence

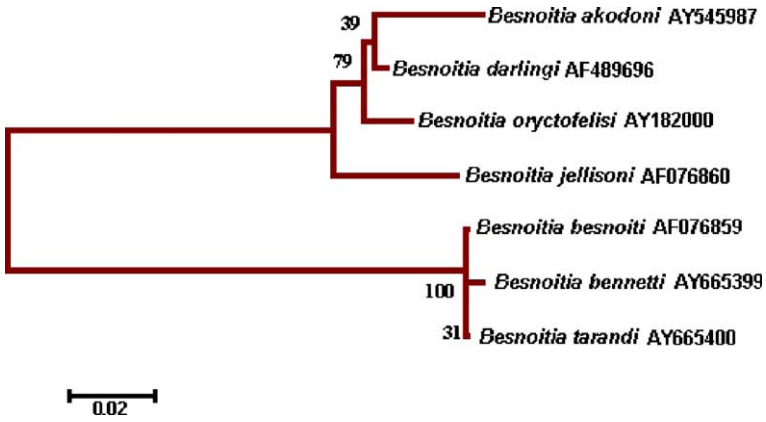

Fig. 9. Consensus of 500 bootstrap replicate trees reconstructed under the minimum evolution criterion based on Kimura 2-parameter distances, using an alignment composed of 250 bp of ITS-1.

or absence of enigmatic bodies. Neither of two domestic cats fed 25–100 *Besnoitia* sp. tissue cysts shed oocysts.

It remains uncertain whether one or more *Besnoitia* species are present in roe deer, mule deer, and musk ox, which belong to different genera. It is also uncertain whether the same parasite affects both reindeer and caribou. Therefore, we designate the reindeer (*R. tarandus tarandus*) as the type intermediate host for *B. tarandi*.

In general, the structure of tissue cysts of *B. tarandi* found in the present study is essentially similar to that of *B. oryctofelisi*. Dubey and Lindsay (2003) described the development and structure of *B. oryctofelisi* tissue cysts in experimentally-infected animals. They provided evidence that the outer two layers of the tissue cyst walls were of host origin, as they did not stain with the *B. oryctofelisi* antibodies. However, the inner (third) layer and the bradyzoites were stained with the antibodies indicating parasitic origin. The results of immunohistochemical staining with *B. tarandi* tissue cysts in the present study confirm the findings of Dubey and Lindsay (2003).

Until recently, enigmatic bodies, first described in *B. jellisoni* by Senaud (1969), were considered characteristic for *Besnoitia* spp. bradyzoites. They contain a central core enclosed in a membrane; their function is unknown. However, extensive studies failed to identify enigmatic bodies in bradyzoites of *B. besnoiti* of cattle (Dubey et al., 2003b), nor were they previously observed in *B. bennetti* of equids (van Heerden et al., 1993). No enigmatic bodies were identified in bradyzoites of *B. tarandi* we studied here, nor were they reported in either of two ultrastructural studies of Canadian reindeer *Besnoitia* species (Glover et al., 1990; Ayroud et al., 1995). In the study of *B. tarandi* from Sweden (Hilali et al., 1990), structures labeled as enigmatic bodies lack their characteristic outer membrane and a central core. Fixing tissues using either formalin or glutaraldehyde seems to preserve the enigmatic bodies. Therefore, it is unlikely that the fixative (formalin) used in the present study caused this difference. We therefore conclude that *B. tarandi*, like *B. bennetti* and *B. besnoiti*, lacks enigmatic bodies.

In *B. tarandi*, the rhoptries were as long as the bradyzoites and in some, the bulbous end was directed towards the conoidal end instead of the non-conoidal end. To our knowledge, this observation has not been made previously in bradyzoites of any coccidian except *T. gondii* (Dubey et al., 1998). Ultrastructurally, *B. tarandi* bradyzoites appeared to possess ~50 micronemes. This is in contrast to the higher number of micronemes (~300) found in the bradyzoites of *B. darlingi* (Dubey et al., 2002), *B. oryctofelisi* (Dubey et al., 2003a) and *Besnoitia akodoni* (Dubey et al., 2003c).

Cutaneous besnoitiosis attributed to *B. besnoiti* exacts substantial economic loss among cattle in Africa, Israel, and some parts of Europe (Dubey et al., 2003a). Although besnoitiosis in goats was presumed to be caused by a distinct taxon, *B. caprae*, an absence of diagnostic variation in the ITS-1 between caprine isolates and those from cattle and wildebeest has questioned this distinction (Ellis et al., 2000). In order to verify the placement of the *B. tarandi* isolate within a monophyletic group comprising other named *Besnoitia* congeners, and in order to evaluate the degree to which it could be differentiated from such congeners, three portions of nuclear ribosomal DNA were characterised and compared to other available homologues. Because different subsets of sequences were available for comparison at each locus, separate alignments were constructed for each, using ClustalW, and subjected to independent phylogenetic reconstruction. Here, we have further established the phylogenetic affinity of *Besnoitia* isolates from caribou to that of cattle and equids. The absence of enigmatic bodies in the above three species of *Besnoitia* appears to provide a morphologic validation to the molecular data.

Developing adequate laboratory models would promote the study of the biology and epidemiology of ungulate besnoitiosis. Tissue cysts of *B. tarandi* have not yet been produced in laboratory animals or in cell culture. However, the parasite was lethal to interferon-gamma gene knock out (KO) mice irrespective of the route of inoculation Gerbils

that are susceptible to experimental infections with other species of *Besnoitia* (*B. darlingi*, *B. besnoiti*, *B. oryctofelisi*, and *B. jellisoni*) do not become infected by *B. tarandi* by subcutaneous, intraperitoneal or oral routes. Limited trials have also revealed that, whereas rabbits are refractive to infection with *B. tarandi* and *B. darlingi*, they could be infected with *B. besnoiti* and *B. oryctofelisi*. Out-bred Swiss Webster mice were also refractive to infection with *B. tarandi*. However, inoculation of even a few tachyzoites resulted in fatal acute besnoitiosis in transgenic mice lacking interferon-gamma, a critical component of T-cell mediated immunity. Route of infection or dose of parasite did not influence the outcome in KO mice. Although useful as a bioassay for isolation of parasite, the outcome renders KO mice of limited value in studying the pathogenesis and immunology of such infections in their natural intermediate hosts.

Domestic cats serve as definitive hosts for the three species of *Besnoitia* whose life cycles are known (*B. darlingi*, *B. oryctofelisi*, and *B. wallacei*). Results of the present study and those reported by Glover et al. (1990) and Ayroud et al. (1995) indicate that domestic cats do not play this role for *B. tarandi*. Epidemiologic evidence argues against congenital transmission of *Besnoitia* species. How reindeer and caribou become infected remains a mystery; transmission by large members of Felidae (cougars, lynx) remains a possibility. Reduction of exposure to biting insects appeared to reduce the transmission of besnoitiosis within the Assinibione Park Zoo (Glover et al., 1990), suggesting the possibility that such arthropods may be capable of mediating mechanical transmission among ungulates. Such a transmission route, if significant to parasite dissemination and persistence, would depart significantly from the dependency that tissue cyst forming coccidia are typically thought to place on vertebrate carnivores. Besnoitiosis in reindeers causes economic losses to meat industry. Hence it is essential to have a better understanding of the biology and life cycle of this parasite, including the possible definitive host(s), to formulate adequate control measures.

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